

Exhibit B

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Genetics

Highly efficient germ-line transmission of proviral insertions in zebrafish

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ABSTRACT An important technology in model organisms is the ability to make transgenic animals. In the past, transgenic technology in zebrafish has been limited by the relatively low efficiency with which transgenes could be generated using either DNA microinjection or retroviral infection. Previous efforts to generate transgenic zebrafish with retroviral vectors used a pseudotyped virus with a genome based on the Moloney murine leukemia virus and the envelope protein of the vesicular stomatitis virus. This virus was injected into blastula-stage zebrafish, and 16% of the injected embryos transmitted proviral insertions to their offspring, with most founders transmitting a single insertion to ~2% of their progeny. In an effort to improve this transgenic frequency, we have generated pseudotyped viral stocks of two new Moloney-based genomes. These viral stocks have titers up to two orders of magnitude higher than that used previously. Injection of these viruses resulted in a dramatic increase in transgenic efficiency; over three different experiments, 83% (110/133) of the injected embryos transmitted proviral insertions to 24% of their offspring. Furthermore, founders made with one of the viruses transmitted an average of 11 different insertions through their germ line. These results represent a 50- to 100-fold improvement in the efficiency of generating transgenic zebrafish, making it now feasible for a single lab to rapidly generate tens to hundreds of thousands of transgenes. Consequently, large-scale insertional mutagenesis strategies, previously limited to invertebrates, may now be possible in a vertebrate.

Traditionally, the generation of transgenic zebrafish has been achieved by the microinjection of plasmid DNA into the cytoplasm of the one-cell stage embryo (1-3). Although this method is useful, efficiency is variable, and transgenes are frequently present in tandem arrays and can have complex unpredictable structures (1, 3). More recently, retroviral infection has emerged as a method for generating transgenic zebrafish (4). In initial studies, Burns *et al.* (5) demonstrated that a pseudotyped retroviral vector, containing a genome based on the Moloney murine leukemia virus (MLV) and the envelope glycoprotein (G-protein) of the vesicular stomatitis virus (VSV), was able to infect a cultured zebrafish cell line. This result was important because previously the host range of the standard retroviral vectors did not permit infection of fish cells (5), and as a result the zebrafish was inaccessible to retroviral vector technology.

Subsequently, our laboratory showed that retroviruses pseudotyped with the VSV G-protein are able to infect the zebrafish germ line following injection of a concentrated stock of an MLV/VSV-pseudotyped virus into blastula-stage zebrafish embryos (4). In these studies, 16% (8/51) of the potential founders tested transmitted proviral insertions to 2-3% of their F1 progeny, with founders transmitting 1-2

different insertions. These results suggested that pseudotyped retroviral vectors could be useful tools for generating transgenic zebrafish and that, if the transgenic frequency could be increased substantially, they might also prove to be effective insertional mutagens.

To determine if we could improve the efficiency of generating transgenic fish using retroviral vectors, we constructed two new MLV-based genomes and generated viral stocks from these constructs with titers up to two orders of magnitude higher than the previously used viral stock (4). Injection of these new viral stocks into blastula-stage embryos resulted in as much as a 50- to 100-fold increase in the efficiency of generating transgenic insertions as compared with previously obtained results using either plasmid microinjection (3) or retroviral infection (4). These results suggest that the efficiency of generating transgenic zebrafish using pseudotyped retroviral vectors is correlated with the titer of the viral stock *in vitro* and that, at the highest titer we have tested to date, the germ line of every injected fish can harbor many different proviral integrations. Consequently it is now feasible for a small lab to generate tens to hundreds of thousands of proviral transgenes in zebrafish. This work represents a major advance in transgenic technology in zebrafish and may make large-scale insertional mutagenesis and the rapid identification of phenotypically interesting genes possible in this vertebrate system.

MATERIALS AND METHODS

Plasmids Used to Make Retroviral Constructs. pSFG (ECT-) (obtained from R. Mulligan, Massachusetts Institute of Technology) contains deletions in the U3 region of the 3'-long terminal repeat (LTR) that remove the MLV transcriptional regulatory elements. The *Xenopus* eF1 α promoter (6) was placed into the 3'-LTR, and the resulting construct, pSFG-nlacZ (SFG; see Fig. 1A), was expected to generate proviruses with this promoter driving a nuclear localized *E. coli* β -galactosidase (*lacZ*) gene (see Fig. 1B). pNK-lacZ (NK; see Fig. 1A) was derived from pLZRNL (7). The eF1 α promoter was placed upstream of the *lacZ* gene, and the RSV LTR and neomycin phosphotransferase gene were removed.

Generation of Stable Retroviral Producer Clones. SFG and NK were each transfected into a 293 gag-pol packaging cell line (293GP; obtained from Viagene, San Diego) with a construct containing the puromycin acetyltransferase gene driven by the simian virus 40 early promoter and with an MLV LTR providing the polyadenylation signal (see Fig. 1A). The packaging cell line used does not express any envelope protein but does express the gag-pol protein required to make infectious retroviral core particles (5). Puromycin-resistant cell clones were screened for virus production by transient transfection of a construct expressing the VSV G-protein from the human cytomegalovirus promoter and subsequent titering on

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Abbreviations: VSV, vesicular stomatitis virus; MLV, murine leukemia virus; G-protein, glycoprotein; LTR, long terminal repeat; SFG, pSFG-nlacZ; NK, pNK-lacZ; cfu, colony-forming unit(s).

mouse 3T3 cells. This construct, pHCMV-G (8), provides the envelope protein necessary to produce infectious pseudotyped virus, and such virus is indicated with the designation (G).

Titering was performed by infecting mouse 3T3 cells and zebrafish PAC2 cells (ref. 4; P. Culp and N.H., unpublished data) with serial dilutions of virus and then staining 48 hr later with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) to detect lacZ activity in infected cells. An SFG(G)-producing clone that produced titers of $5-10 \times 10^6$ colony-forming units (cfu)/ml on 3T3 cells and 1×10^4 cfu/ml on zebrafish PAC2 cells was identified. The SFG(G) virus was unexpectedly found by Southern blot to be a mixture of three different viral genomes, representing recombinants of the plasmids used to construct the virus-producing cell line (see Results). An NK producer clone that produced lacZ titers of $5-10 \times 10^6$ cfu/ml on 3T3 cells and 1×10^5 cfu/ml on PAC2 cells was identified. Southern blot analysis indicated that virus from this clone produces the expected proviral genome (data not shown).

Virus-containing supernatant from the selected SFG and NK clones was concentrated as described (5, 8) to lacZ titers of $1-2 \times 10^9$ cfu/ml on 3T3 cells. The lacZ titers of the concentrated SFG(G), and NK(G) stocks on PAC2 cells were $5-10 \times 10^6$ cfu/ml and $1-2 \times 10^8$ cfu/ml, respectively. Previous studies in our lab have indicated that lacZ may be a substantially less effective reporter in zebrafish PAC2 cells than in mouse 3T3 cells (P. Culp and N.H., unpublished data). Therefore the relative titer of these stocks on 3T3 and PAC2 cells was estimated by comparing the amount of integrated proviral DNA in both cell types after infection with the same dilution of a virus stock. For both SFG(G) and NK(G), the amount of integrated proviral DNA in PAC2 cells was found to be roughly two-fold less than that in 3T3 cells (data not shown). The lacZ titers of the concentrated SFG(G) and NK(G) stocks used for injection into embryos were 2×10^9 cfu/ml on 3T3 cells and were therefore estimated to be $\sim 1 \times 10^9$ cfu/ml on PAC2 cells.

Generation and Identification of Transgenic Founder Fish. Ten to 20 nanoliters of the concentrated SFG(G) and NK(G) viral stocks containing 8 μ g of polybrene per ml were injected into 4–5 locations among the blastomeres of blastula-stage zebrafish embryos ($\sim 512-2000$ cell stage). Injected embryos were raised to sexual maturity and mated either to each other or to wild-type fish. Genomic DNA was prepared from pools of the F1 progeny as described (4) and was tested by the polymerase chain reaction (PCR) for the presence of proviral DNA. The nucleotide sequence of the primers used to detect both SFG and NK founders (primer set 1; see Fig. 1A) is as follows. The upstream primer sequence is 5'-ATATCGACG-GTTTCATATGGG-3' and is within the coding sequence of the lacZ gene. The downstream primer sequence is 5'-GTACTCTATAAGGCTTCAGCTGG-3' and is within the MLV-derived sequences downstream of the lacZ gene. This set of primers amplifies a 232-bp sequence in SFG and about a 200-bp sequence in NK. Primers designed to detect sequences within the zebrafish wnt5a gene were used as internal controls in each reaction and are the same as those previously described (4). The PCR program used was as follows: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 32 cycles, with an initial denaturation step at 94°C for 2 min and a final elongation step at 72°C for 5 min.

Identification of Transgenic F1 Fish. The F1 progeny of founders were raised and individual genomic DNA samples were prepared from caudal fin clips by incubation in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.2 mg of proteinase K per ml for at least 3 hr at 55°C. PCR was then used to detect the presence of proviral sequences. Primer set 1 (see Fig. 1A) was used to identify transgenic progeny from NK founders, while a second set of primers, primer set 2 (see Fig. 1A), was used to identify transgenic progeny from SFG founders. Primer set 2 was designed when it was found that the SFG(G)

stock generated three different proviruses, two of which were not detectable by primer set 1 (see Results). The nucleotide sequence of primer set 2 is as follows. The upstream primer sequence is 5'-ATCCTCTAGACTGCCATGG-3' and includes the start codon of the lacZ gene. The downstream primer sequence is 5'-ATCGTAACCGTGCATCTG-3' and is within the coding sequence of the lacZ gene. This set of primers amplifies about a 228-bp sequence. All identified transgenic offspring from a single founder were kept together, and subsequently tail DNA was reisolated from these fish for Southern blot analysis.

Southern Blot Analysis. Genomic DNA was digested with the indicated restriction enzymes, electrophoresed through a 0.8% agarose gel and blotted to Hybond N⁺ nylon membranes (Amersham). Radiolabeled probes were made using the Random Primed DNA Labeling Kit (Boehringer Mannheim). Hybridizations were carried out at 65°C in a Robbins Scientific Model 2000 Hybridization Incubator (Robbins Scientific, Mountain View, CA) in a solution containing 0.25 M Na₂HPO₄ (pH 7.4), 1 mM EDTA, 10 mg of BSA per ml, and 7% SDS. Filters were washed 3 times for 20 min each with 0.1× SSC and 0.1% SDS at 65°C.

RESULTS

High Frequency Germ-Line Transmission of Proviral Integrations. Two pseudotyped viruses, SFG(G) and NK(G), were constructed for these studies and have MLV-based genomes (Fig. 1A) and an envelope containing the VSV G-protein. These viruses are similar to the MLV/VSV-pseudotyped virus previously shown to be capable of stably integrating proviral DNA into the zebrafish genome (4). Concentrated stocks of SFG(G) and NK(G) were prepared from stable producer cell lines and were titered on both mouse 3T3 cells and zebrafish PAC2 cells. Because of complications in determining the titer of these viruses on PAC2 cells (see Materials and Methods), only the titers on 3T3 cells will be given hereafter.

The SFG(G) virus stock (2×10^9 cfu/ml on 3T3 cells) was microinjected into zebrafish embryos at about the 1000-cell stage. Following microinjection, the embryos were incubated at either 26°C or 28°C. Although many embryos (50–80%) did not survive to the next day or were malformed, the majority of those that appeared normal at 24 hr grew to adulthood. To detect germ-line transmission of proviral DNA, the injected embryos were raised to adulthood and mated, and genomic DNA from pools of 24-hr F1 embryos was tested for the presence of proviral sequences by PCR. As shown in Table 1, 90/106 (85%) of the potential SFG founders tested (experiments 1 and 2) were found to transmit proviral DNA to their F1 progeny.

Although aliquots of the same virus stock were used in both SFG(G) experiments listed in Table 1, the frequency of germ-line transmission in the first experiment, 71% (40/56), is significantly lower than the 100% (50/50) obtained in the second experiment. The primary difference between these two experiments was the temperature the embryos were incubated at after injection, suggesting that viral infection occurs more efficiently at 28°C than at 26°C. We have obtained similar results that support this conclusion using two other MLV/VSV viruses (N.G., M.A., and N.H., unpublished data).

Initial injections with an undiluted stock of the virus NK(G) (2×10^9 cfu/ml on 3T3 cells) resulted in all injected embryos being dead or severely malformed by the next day. Four-fold dilutions of the concentrated NK(G) stock, to 5×10^8 cfu/ml on 3T3 cells, resulted in survival rates similar to those observed using SFG(G) and were used to generate the potential NK founders. Of 27 potential NK founders tested, 20 (74%) were found to transmit proviral DNA to their F1 progeny (Table 1).

The toxicity observed upon injection of some viral stocks into blastula-stage embryos could be a function of the fuso-

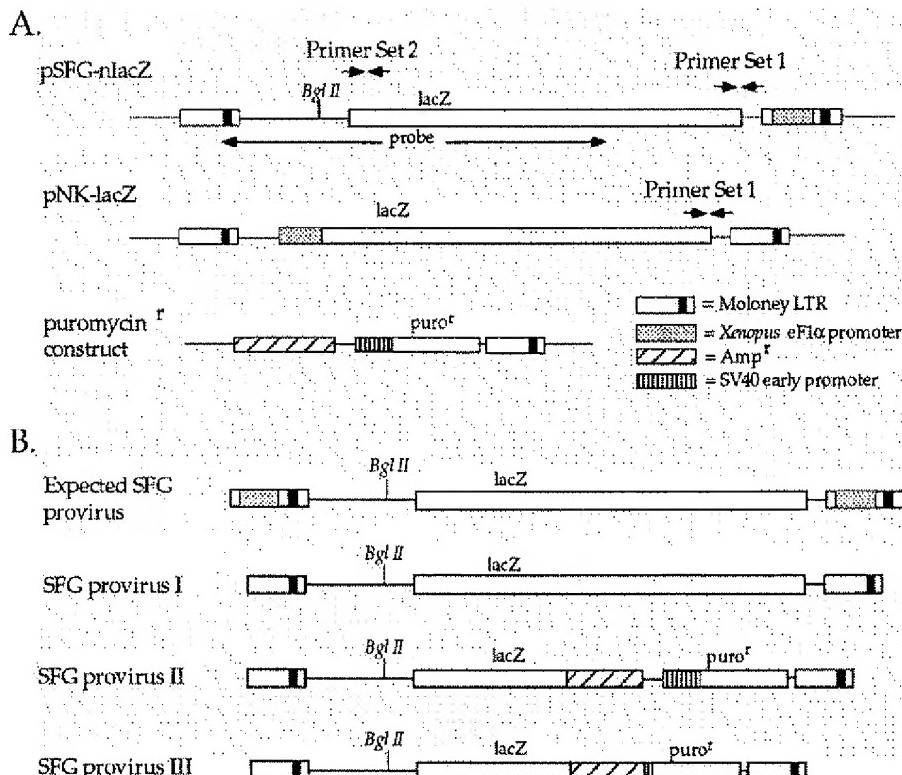


FIG. 1. Schematic representation of the plasmids used and of the predicted and observed proviral structures. (A) Maps of SFG (with 3'-LTR modified to contain the *Xenopus* eFlα promoter), NK, and the construct used to confer resistance to puromycin. The locations of primer sets 1 and 2 are indicated, as is the region of SFG that was used as a probe for Southern blots. (B) The expected SFG proviral genome, and the actual SFG proviral genomic structures.

genic nature of the VSV G-protein (9). Due to the inherent variability in the efficiency of transient transfection of the VSV-G expressing plasmid, a step required to make MLV/VSV-pseudotyped viruses (see ref. 8), the amount of VSV G-protein may vary between virus stocks. Such variability could account for the difference in toxicity seen between the SFG(G) and NK(G) viruses used here, although contaminants in the viral stocks, derived from the producer cells used to make the virus, could also be responsible.

Table 1. High-frequency germ-line transmission of proviral insertions

Exp.	Virus injected	Temp.*	Titer on 3T3s, cfu/ml†	Potential founders tested	Founders identified	
				No.	% No.	
1	SFG(G)	26°C	2 × 10 ⁹	56	40	71
2	SFG(G)	28°C	2 × 10 ⁹	50‡	50	100
3	NK(G)	28°C	5 × 10 ⁸	27	20	74

Injected embryos were raised, and genomic DNA was isolated from pools of their F1 progeny and tested by PCR for the presence of proviral DNA. In some cases, F1 pools were not tested in this manner; instead, F1 embryos were raised and tested by isolating DNA from fin clips.

*The temperature at which embryos were incubated for 24 hr after injection.

†The titer on 3T3 cells is shown for comparison because an accurate determination of SFG(G) titer on PAC2 cells was complicated by limited or no lacZ expression (see Materials and Methods).

‡In experiment 2 a total of five potential founders with between 8 and 21 F1 progeny tested were found to be negative but were considered inconclusive, due to the small number of progeny tested, and are not listed.

Germ-Line Mosaicism of Founders. To identify individual transgenic F1 fish without sacrificing them, genomic DNA was isolated from caudal fin clips of 6- to 8-week-old F1 progeny of identified founders and tested by PCR for the presence of proviral DNA. As shown in Fig. 1B and as discussed below, the SFG(G) virus stock contains a mixture of three viral genomes. Primer set 2 (see Fig. 1A), which detects all three SFG proviruses, was used to detect F1s transgenic for SFG proviruses. Primer set 1 (see Fig. 1A) was used to detect F1s transgenic for NK proviruses.

The percentage of transgenic offspring from SFG founders ranged from 12 to 70% with an average of 29% (Table 2). The percentage of transgenic offspring from NK founders was somewhat lower and ranged from 3 to 28%, with an average of 13%. A plausible explanation for the difference in both the frequency of germ-line transmitting founders (see above) and the frequency of transgenic F1s from these founders between the SFG(G) and NK(G) injections would be that the SFG(G) stocks used for injection were 4-fold higher in titer than the NK(G) stock used (see Table 1).

SFG Founders Transmit an Average of 11 Proviral Insertions to Their F1 Progeny. To examine the number of different insertions being transmitted through the germ line of the founder fish, Southern blot analysis was performed on genomic DNA from fin clips of individual transgenic F1s from SFG founders. The DNA was digested with *Bgl*II, which cuts once within all three SFG proviral genome types (see Fig. 1B and below), and analyzed by Southern blot. Depending upon the location of *Bgl*II sites in the genomic DNA adjacent to the insertion, each different insertion was expected to yield two junction fragments of diagnostic sizes. An example of a Southern blot used to compare insertions is shown in Fig. 2.

Table 2. Analysis of germ-line transmission of proviral insertions from individual SFG and NK founders

Founder	Transgenic F1 progeny, %	No. of insertions transmitted to F1s
SFG1	12%	—
SFG8	39%	—
SFG17	23%	—
SFG26	19%	—
SFG35	70%	11
SFG48	23%	5
SFG49	28%	7
SFG51	39%	7
SFG52	36%	12
SFG54	21%	6
SFG57	20%	14
SFG59	56%	14
SFG62	24%	12
SFG64	12%	12
SFG66	33%	10
SFG67	30%	10
SFG69	25%	19
SFG73	17%	9
SFG77	57%	22
SFG80	21%	6
SFG81	13%	6
SFG89	28%	12
Average	29%	11
NK3	24%	—
NK4	24%	—
NK5	3%	—
NK6	8%	—
NK7	7%	—
NK10	4%	—
NK11	28%	—
NK12	12%	—
NK16	15%	—
NK20	9%	—
Average	13%	—

Transgenic F1s were identified by isolating genomic DNA from caudal fin clips and testing by PCR for proviral sequences. Genomic DNA from identified transgenic F1 fish was then digested with *Bgl*II, which cuts once in the proviral sequence, and Southern blot analysis was performed. Junction fragment sizes were compared between fish, and those with identical patterns were classified as having the same insertion.

The proviral insertions in 19 transgenic offspring from founder SFG77 were compared. Among these fish, 14 have one insertion, 4 have two insertions, and 1 has three insertions (lane 6), with some insertions being present in more than one fish. In total these fish harbor 16 different insertions among them.

Progeny from 18 outcrossed founders were analyzed by Southern blot and were found to contain 194 different insertions, indicating that on average each founder transmits 11 different insertions to its F1 progeny (see Table 2). Although the majority of transgenic F1 fish (65%) were found to have 1 proviral insertion each, individual F1 fish were frequently found with 2, 3, or 4 different proviral insertions (26%, 7%, and 2%, respectively). The mosaicism of individual insertions in the germ line of founders varied, with some insertions being present in <1% of the F1 progeny from a given founder, and others being present in as much as 14% of the F1 progeny from a given founder. Of 187 insertions analyzed, ~30% were found to be transmitted to at least 3% of the founder's progeny.

Analysis of SFG Proviral Genome Structures. As mentioned above, the SFG(G) virus stock contains three different viral genomes. To examine the structures of these proviruses in transgenic fish, genomic DNA from individual fish harboring the different proviruses was digested with various combina-

tions of the following restriction enzymes: *Xba*I, *Pvu*II, *Eco*RV, *Eco*RI, *Bam*HI, *Nhe*I, *Hinc*II, *Sac*I, *Hind*III, *Nco*I, *Sph*I, and *Dra*I. Based on Southern blot analysis of these digests, restriction maps that were consistent with every digest examined were constructed. Structural maps of the three SFG proviral genomes (SFG provirus types I, II, and III) are shown in Fig. 1B. All three SFG proviral genomes were found to have wild-type MLV LTRs. This result was surprising because, based on the plasmid used to generate the SFG producer cell line (pSFG-nlacZ, see Fig. 1A), SFG proviruses were expected to possess modified LTRs with the *Xenopus eFlα* promoter in the U3 region (see Fig. 1B). In addition to the unexpected LTR structure found, two of the three SFG proviral genomes (types II and III) were found to have large deletions in the *lacZ* gene and to contain sequences from the puromycin resistance construct, which had been used to allow drug selection of stable producer clones (see Materials and Methods and Fig. 1A). The absence of the desired viral genome in the SFG(G) stocks, and the presence of the three different viral genomes in these stocks is likely to be the result of DNA rearrangements that occurred during the transfection of pSFG-nlacZ and the puromycin resistance construct into the packaging cell line.

DISCUSSION

The results presented here demonstrate that MLV/VSV-pseudotyped retroviral vectors can be used to generate transgenic zebrafish with extremely high efficiency. At previous transgenic frequencies, using either DNA microinjection or retroviral infection, the generation of 100 potential transgenic founders would typically result in the germ-line transmission of 10–20 transgenes (3, 4). The current work represents a dramatic improvement in transgenic frequency and shows that it is now possible, using retroviral infection, for 100 potential founders to result in the germ-line transmission of 1000 transgenes. One possible explanation for the increased transgenic frequency seen here as compared with previous results using the pseudotyped retrovirus LZRN(L) is that the titers of both the SFG(G) and NK(G) stocks used here were ~100-fold higher than that of the LZRN(L) stock used previously (4).

Based upon the transgenic frequencies reported here, large-scale insertional mutagenesis, a technique that has not been practical in vertebrate model systems, may now be possible in zebrafish. Although large-scale chemical mutagenesis can be performed in zebrafish (10, 11) and has yielded many interesting mutants, an insertional mutagenesis strategy could be a powerful alternative. This is because mutagenic insertions provide a molecular tag to facilitate the cloning of mutated genes, circumventing the laborious positional cloning methods often required to clone chemically mutated genes.

In fruit flies, insertional mutagenesis is possible because P-elements can be used to generate many thousands of insertions that can be screened for integration events of interest (12). While previously the generation of many thousands of transgenic insertions was theoretically possible in vertebrate systems such as the mouse and the zebrafish, the resources and time required to do so were prohibitive. The present work, however, indicates that it is now feasible for a single lab to rapidly produce as many as 100,000–200,000 transgenes in zebrafish. This could be achieved by generating 10,000–20,000 founders, each of which would transmit 10 insertions to its F1 progeny as shown here (Table 2). We estimate that it would take 4–6 people roughly three months to generate the founders.

Based upon the size of the zebrafish genome (1.6×10^9 bp), a screen involving 200,000 insertions would have, on average, one insertion every 8 kb. If the average gene spans ~10 kb, then a screen of this size would be expected to have potentially mutagenic insertions into most of the genes in the genome. The

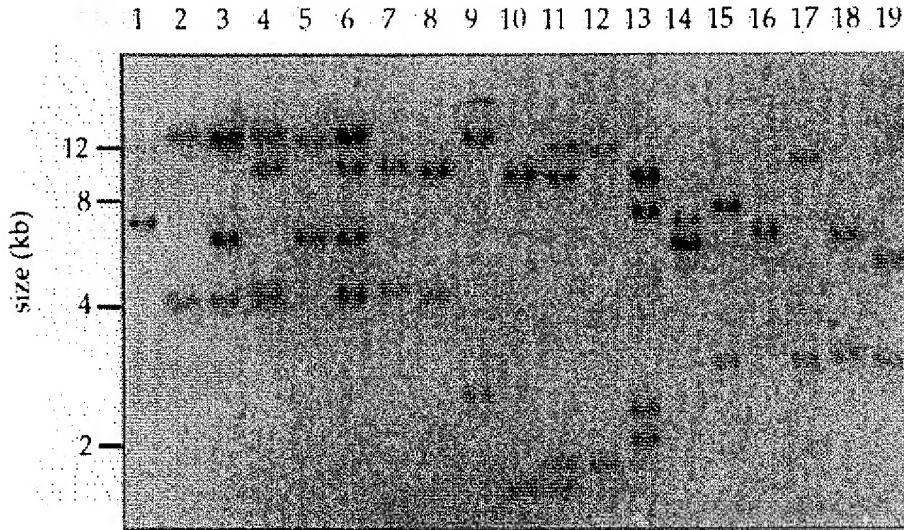


FIG. 2. Southern blot analysis of DNA from transgenic F1 progeny of founder SFG77. The genomic DNA was digested with *Bgl*II and probed with the sequence indicated in Fig. 1A. Each insertion is expected to produce two junction fragments with sizes characteristic of the site of integration. The result of segregation during meiosis is apparent in lanes 2–7 and 10–12. For example, the F1 represented in lane 11 has four bands representing two insertions. These two insertions can be seen independently in the F1s represented in lanes 10 and 12.

actual efficiency of such a screen would depend upon whether or not proviruses integrate randomly into the zebrafish genome and upon the mutagenicity of proviral DNA integrated into zebrafish genes. If, for example, proviral insertions into the zebrafish genome preferentially occur into intergenic sequences, then the likelihood of integrating into and mutating genes would be greatly reduced. However, studies of retroviral integration in the mouse and chicken suggest that proviral insertions occur either at random (13) or possibly with a preference for transcribed regions of the genome (14, 15). Preliminary results from our lab indicate that proviruses can and do integrate into single-copy sequences, as well as transcribed regions of the zebrafish genome (K.K., N.G., and N.H., unpublished data).

There are several possible ways a large-scale insertional mutagenesis screen in zebrafish might be conducted. One strategy would simply be to generate insertions, breed them to homozygosity, and screen for mutant phenotypes. Although such a screen is labor-intensive and limited by the time and space required, it should be possible to screen several thousand insertions in this manner. It is unknown how many mutants would be generated in a screen of this size, because the number of proviral insertions into the zebrafish genome required to produce a mutant phenotype remains to be determined. We are currently conducting a pilot screen to determine this number. In mice, 5% of proviral insertions disrupt essential genes (16).

The inbreeding strategy mentioned above might be an effective way to isolate insertional mutants, although it is limited by the need to maintain very large numbers of individual lines. As a result, using such a strategy a lab could only screen a small fraction of the hundreds of thousands of transgenes that could be generated. An alternative strategy, which would permit the screening of a much larger number of insertions, would be to screen haploid embryos. Haploid zebrafish embryos are easy to generate and undergo relatively normal early development (17). Transgenic F1 fish, heterozygous for proviral insertions, could be used to generate haploid F2 embryos to be screened for mutant phenotypes. A phenotype observed in 50% of the haploid embryos would indicate the presence of a mutagenic insertion.

Another possible approach to insertional mutagenesis in zebrafish would be to use retroviral gene traps. Gene traps are

constructs containing a reporter gene that can only be expressed after integration into a transcribed endogenous gene (18). Such "activated" integrations are likely to disrupt the function of the gene into which they have integrated and typically express the trap reporter in a temporal and spatial pattern similar to that of the endogenous gene (19, 20). The generation of 20,000 founders, which could be maintained in 100 15-gallon fish tanks, would permit the screening of 200,000 gene trap insertions. F1 embryos from founder matings could be screened for trap activations, and those containing expression patterns of interest could be isolated for further study. If gene traps are activated in fish cells at efficiencies similar to those in mice (18), then 200,000 gene trap insertions in zebrafish could contain as many as 20,000 activated trap patterns, a significant percent of which would be expected to have a mutant phenotype when bred to homozygosity (19).

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